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Changes in the V_H Gene Repertoire of Developing Precursor B Lymphocytes in Mouse Bone Marrow Mediated by the Pre-B Cell Receptor

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Summary

The V_H repertoire on both H chain alleles of normal and λ5-deficient B lineage cells were analyzed by single-cell PCR. The μH chains were tested for their capacity to form a pre-B cell receptor. In bone marrow, D-proximal V_H genes were found preferentially expressed in λ5-deficient pre-B cells and in a newly identified early c-kit$^+$ cytoplasmic μH chain$^+$ pre-B cell population of normal mice. Only half of the μH chains expressed in these cells have the capacity to form a pre-B cell receptor. Representation of the D-proximal V_H genes was found suppressed on the productive but not on the nonproductive V_HDJ_H rearranged alleles of c-kit$^-$ preB-II cells and splenic λ5-deficient B cells. More than 95% of the μH chains expressed in preB-II cells can form a pre-B cell receptor. These results demonstrate that the pre-B cell receptor in normal mice and the B cell receptor in λ5-deficient mice mediate a shift in the V_H repertoire.

Introduction

During B cell development, immunoglobulin (Ig) variable regions of the heavy (H) chain are assembled in a stepwise fashion (Tonegawa, 1983). First, D segments are rearranged to J_H segments on both alleles of the H chain locus (Alt et al., 1984). In the bone marrow of a mouse, this occurs in CD19$^+$ CD45R (B220)$^+$ B-lineage precursors, called preB-I cells, which are c-kit$^+$ CD25$^-$ and express the V_{preB} and λ5 genes, which code for the surrogate light (SL) chain (Melchers et al., 1993; Rolink et al., 1994; Ten Boekel et al., 1995). Next, V_H segments are rearranged to DJ_H-rearranged segments. V_HDJ_H rearrangements are found in c-kit$^-$ CD25$^+$ large cycling preB-II cells, all of which express cytoplasmic μH (cμH) chains, 20% of them as μH chain/SL chain–containing pre-B cell receptors (preBcRs) on their cell surface (Rolink et al., 1994; ten Boekel et al., 1995; Winkler et al., 1995). Hence, preB-II cells are greatly enriched for productively V_HDJ_H-rearranged H chain loci (Rolink et al., 1994). This positive selection of μH chain–expressing pre-B cells, which occurs during proliferative expansion of preB-II cells, is abolished in mice that are defective for preBcR expression, such as the λ5-deficient (λ5T) mice (Kitamura et al., 1992; Rolink, et al. 1993; Karasuyama et al., 1994).

The diversity of 14 families of V_H, 13 functional D, and 4 J_H segments that randomly rearrange with each other, and the added diversity introduced by N-region insertions into the V_H-to-D and D-to-J_H joints, provide an immense "potential" repertoire of different μH chains in preBcRs (Brodeur and Riblet, 1984; Winter et al., 1985; Ichihara et al., 1989; Kofler et al., 1992; Feeney and Riblet, 1993). However, the V_H, D, or J_H segments represented in pre-B cells and later in the mature sIgM$^+$/sIgD$^+$ repertoire do not proportionally correlate with their representation in germline (Reth et al., 1986b; Lawler et al., 1987; Gu et al., 1991; Carlsson et al., 1992; Chang et al., 1992; Teale and Medina, 1992; Gerstein and Lieber, 1993; Huetz et al., 1993). V_H genes belonging to the V_H7183 and V_HQ52 family are overrepresented early in development, as in fetal liver B cells (Yancopoulos et al., 1984; Perlmutter et al., 1985; Wu and Paige, 1986; Alt et al., 1987; Malynn et al., 1990). In particular, the most D-proximal functional V_H gene segment, V_H81X, which is a member of the V_H7183 family, predominates in repertoires early in ontogeny (Yancopoulos et al., 1984; Marshall et al., 1996). In contrast, peripheral mature B cell populations in adult mice express a V_H repertoire in which the frequency of each V_H family used correlates with its germline complexity (Dildrop et al., 1985; Schulze and Kelsoe, 1987; Jeong and Teale, 1988; Yancopoulos et al., 1988; Sheehan and Brodeur, 1989). A recent report showed a decline of V_H81X representation in V_HDJ_H rearrangements during B cell development in the bone marrow of adult mice (Marshall et al., 1996). Indeed, V_H81X-expressing B cells are rarely found in the spleen (Yancopoulos et al., 1988; Huetz et al., 1993; Decker et al., 1995). Moreover, virtually all V_HDJ_H rearrangements utilizing V_H81X genes in the splenic sIg$^+$ B cell repertoire are nonproductive, while 25%–30% of V_H81X rearrangements in sIg$^-$ pre-B cells in the bone marrow are productive (Decker et al., 1991; Carlsson et al., 1992; Huetz et al., 1993). This is in contrast to rearrangements using other V_H genes, which are largely productive (Decker et al., 1991; Gu et al., 1991; Decker et al., 1995).

Although the V_H repertoire in fetal and neonatal liver and adult splenic B cells has been well studied, the representation of the different V_H families in precursor B cells during B cell development in the adult bone marrow has not been clearly defined. Two reports stated that the expressed V_H repertoire in bone marrow B cells was biased toward the V_H7183 gene family, although to a lesser extent than was observed in B cells early in ontogeny (Freitas et al., 1990; Malynn et al., 1990). However, other studies failed to confirm these findings (Jeong and Teale, 1988; Jeong and Teale, 1989; Miceli and Schulze, 1991). Thus it is still controversial whether pre-B cells in the bone marrow of adult mice have a expressed V_H repertoire similar to that of B cells early in ontogeny. Furthermore, the mechanisms underlying the changes in V_H repertoire between early and peripheral B cells are still unresolved.

Preferential representation of the V_H7183 and V_HQ52 family early in B cell development could be the result of preferential usage of these segments on the genomic level during the rearrangement process (Yancopoulos et al., 1984; Perlmutter et al., 1985; Alt et al., 1987; Jeong

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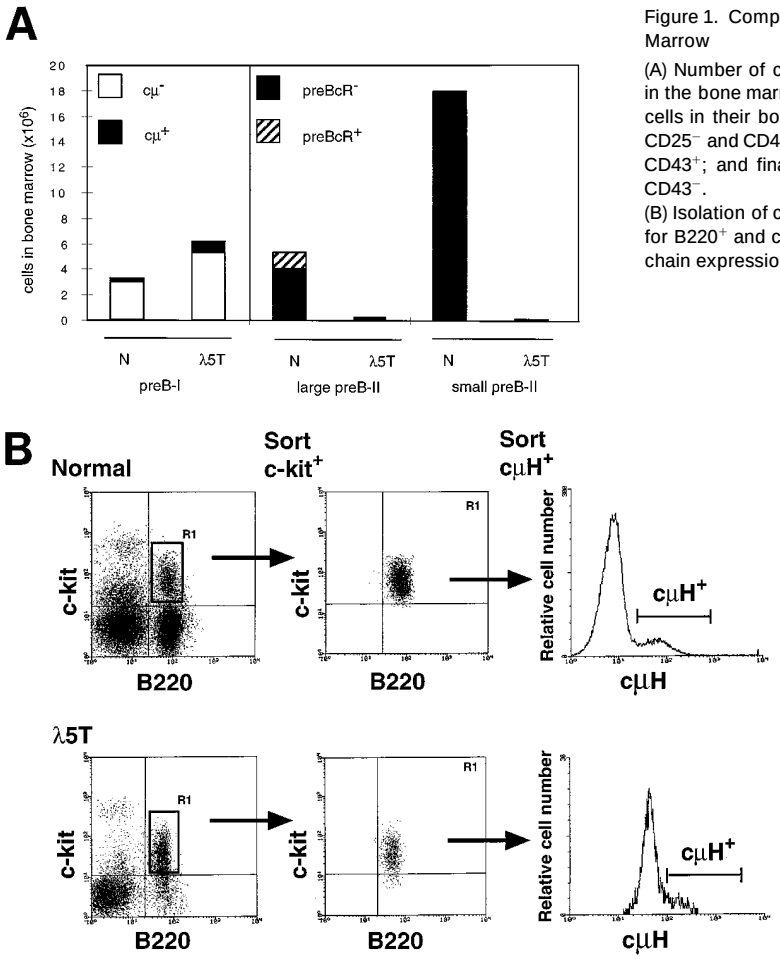

Figure 1. Compartments of Different B Lineage Cells in the Bone Marrow

(A) Number of cells in CD19$^+$ sIg$^-$ B cell precursor compartments in the bone marrow of normal and λ5T adult mice (of 10^8 nucleated cells in their bone marrow). The preB-I cells are defined as c-kit$^+$ CD25$^-$ and CD43$^+$; large preB-II cells are c-kit$^-$ CD25$^+$ and partially CD43$^+$; and finally, the small preB-II cells are c-kit$^-$ CD25$^+$ and CD43$^-$.

(B) Isolation of c-kit$^+$ cμH chain$^+$ B-lineage cells. Cells were sorted for B220$^+$ and c-kit$^+$ and subsequently stained for cytoplasmic μH chain expression.

and Teale, 1988; Malynn et al., 1990). In agreement with this notion, it has been suggested that the recombination signal sequences of V_H7183 mediate recombination more frequently than those of V_HJ558 (Connor et al., 1995). Suppression of the representation of certain V_H segments later during B cell generation could then be the result of the action of the preBcR. In this case, mutant mice defective in preBcR expression, such as the λ5T mice, might not display the change of V_H representation in adult bone marrow. Also, pre-B cells expressing a μH chain that is incapable of pairing with the SL chain would be excluded from the repertoire (Keyna et al., 1995).

In this study we investigate the repertoire of V_H genes in the productively as well as nonproductively rearranged alleles of single pre-B cells of normal and λ5T adult mice in precursor B cell pools that exist before light (L) chain gene rearrangements. This approach allows assessment of the status of rearrangement and the V_H representation in both H chain alleles of precursor B cells during B cell development. We demonstrate that early, cytoplasmic μH chain–expressing pre-B cells have a V_H repertoire that is very similar to that of B cells early in ontogeny, that is, biased toward V_H7183 and V_HQ52. The results indicate that in normal mice the suppression of representation of V_H7183 and V_HQ52 family in the expressed V_H repertoire, as seen in peripheral mature B cells, is mediated by the action of the preBcR. In support of this finding, in λ5T mice this suppression appears to be absent.

Results

V_H Repertoire in Large PreB-II Cells of Normal Mice

It has been reported previously that CD19$^+$ B-lineage cells in the bone marrow can be separated into sIg$^-$ preB-I, large preB-II, small preB-II, and sIg$^+$ immature B cells using the markers c-kit, CD25, cell cycle status, and μH chain expression (Rolink et al., 1994). The numbers of cells in the preB-I and large preB-II compartments in the bone marrow of normal and λ5T mice are shown in Figure 1A. In normal mice, the cells in the large preB-II pool have a productively V_HDJ_H-rearranged H chain locus and are sIg$^-$ CD19$^+$ CD45 (B220)$^+$ c-kit$^-$ CD25$^+$ large, actively cycling cells (Rolink et al., 1994). These B cell precursors have their IgL chain loci in germline configuration (ten Boekel et al., 1995). We know from previous analyses (ten Boekel et al., 1995) that half of the large preB-II cells show V_HDJ_H rearrangements in both H chain alleles (designated here as VDJ/VDJ cells), while the other half show one V_HDJ_H and one DJ_H rearranged allele (VDJ/DJ cells). From the pool of large

Table 1. Size[a] of the V_H Families 7183, Q52, and J558 and V_H Representation in Ig^+ Splenic B Cells in C57BL/6 Adult Mice

V_H Family	Size: no.	V_H Representation[b]
7183	8 (~10%)	7% (4%–10%)
Q52	5 (~7%)	6.5% (3%–10%)
J558	45 (~60%)	62.5% (45%–80%)

[a] Estimated germline V_H family size in C57BL/6 mice (Yancopoulos et al., 1988).
[b] Average taken from data published by Dildrop et al. (1985), Wu and Paige (1986), Yancopoulos et al. (1988), Sheehan and Brodeur (1989), and Freitas et al. (1990).

preB-II cells, single cells were isolated by fluorescence-activated cell sorting (FACS) and analyzed for their configuration of the H chain loci by polymerase chain reaction (PCR), followed by sequencing of the obtained PCR products. The observed V_H representation in the V_HDJ_H rearrangements was compared to that of the expressed V_H repertoire of splenic B cells previously published (Table 1). To distinguish the V_H representation on the two alleles of a single cell, we sequenced only the PCR products derived from cells of which both H chain alleles were detected. This was the case for approximately 15% of all cells tested. The sequence analyses are summarized in Table 2. Among the VDJ/VDJ cells, cells carrying two productively V_HDJ_H-rearranged H chain alleles were detected in both normal and λ5T B-lineage cell populations (and will be described as VDJ^+/VDJ^+ cells; E. t. B., et al., unpublished data).

It is evident that in the large preB-II cells, the V_H7183 and V_HQ52 families are strongly overrepresented on the nonproductive (designated VDJ^-) alleles, considering the number of genes in each V_H gene family in germline as a standard (Table 1). The V_HJ558 family is represented below the standard value (Table 2). In contrast, the representation of V_HJ558 family is increased over the standard value on the productively (VDJ^+) rearranged alleles of VDJ^+/VDJ^- as well as VDJ^+/DJ large preB-II cells. On the other hand, a decrease in the V_H representation of the V_H7183 and V_HQ52 families, in fact toward standard values, is seen on the productively rearranged alleles of both VDJ^+/VDJ^- and VDJ^+/DJ large preB-II cells (Table 2). The V_H representation on the VDJ^+ alleles of preB-II cells is similar to that previously reported in splenic $sIgM^+$ B cells (Table 1). The distinct discrepancy in V_H representation on the productive versus nonproductive rearrangements indicates a role for the gene products, namely μH chains, in the process of shaping the V_H repertoire.

Table 2. V_H Representation in VDJ^+/VDJ^-- and VDJ^+/DJ-Rearranged Large PreB-II Cells[a] of Normal Adult Mice

	VDJ^+/VDJ^- Cells		VDJ^+/DJ Cells
V_H Family	VDJ^+ Allele	VDJ^- Allele	VDJ^+ Allele
7183	5 (10%)	15 (29%)	4 (8%)
Q52	2 (4%)	7 (14%)	6 (12%)
J558	36 (71%)	25 (49%)	32 (65%)
Others	8 (16%)	4 (8%)	7 (14%)

[a] Fifty-one single VDJ^+/VDJ^--rearranged cells and 49 single VDJ^+/DJ-rearranged cells were analyzed (from a pool of three femurs).

Table 3. V_H Representation in VDJ^+/VDJ^-- and VDJ^+/DJ-Rearranged PreB Cells[a] of λ5T Adult Mice

	VDJ^+/VDJ^- Cells		VDJ^+/DJ Cells
V_H Family	VDJ^+ Allele	VDJ^- Allele	VDJ^+ Allele
7183	10 (24%)	15 (36%)	12 (24%)
Q52	6 (14%)	5 (12%)	13 (26%)
J558	20 (48%)	11 (26%)	20 (40%)
Others	6 (14%)	11 (26%)	5 (10%)

[a] Forty-two single VDJ^+/VDJ^- rearranged cells and 50 single VDJ^+/DJ rearranged cells were analyzed (from a pool of three femurs).

V_H Repertoire in Precursor B Cells of λ5T Mice

In the bone marrow of λ5T mice, V_H-to-DJ_H rearrangements do occur. However, cells with a productively rearranged H chain allele do not expand into a normal sized preB-II compartment since the preBcR cannot be properly formed (Kitamura et al., 1992; Rolink et al., 1993). Hence, the sIg^- $CD19^+$ CD45R $(B220)^+$ cytoplasmic expressing $(c\mu^+)$ precursor B cell compartment in these mice is markedly reduced in size. In fact, the majority, more than 90%, of these $c\mu^+$ cells express $c\text{-}kit^+$ on the cell surface (Figure 1A).

Single cells of the $CD19^+$ sIg^- $c\mu^+$ pool were FACS sorted and analyzed for their V_H repertoire. As in normal preB-II cells, about half of the analyzed $CD19^+$ sIg^- $c\mu^+$ bone marrow cells of λ5T mice were VDJ^+/VDJ^- and the other half VDJ^+/DJ rearranged. Further, most (~90%) of these cells have their L chain gene loci in germline configuration (data not shown). The structure of the two H chain alleles was analyzed by PCR, followed by sequencing of the PCR products obtained from those single cells in which the two H chain alleles were distinguished. The sequence analyses are summarized in Table 3.

As in the large preB-II cells of normal mice, in λ5T pre-B cells the V_H7183 and V_HQ52 families are overrepresented while members of the V_HJ558 family are underrepresented on the VDJ^- alleles (Table 3). Surprisingly, a similar overrepresentation of the V_H7183 and V_HQ52 families was found on the VDJ^+ alleles of λ5T precursor B cells (Table 3). Hence, the marked change in the V_H repertoire observed on the VDJ^+ alleles of normal preB-II cells is not seen in mice that cannot express the preBcR. Taken together, these data suggest a role for the preBcR in the process of V_H repertoire changes.

Although the pattern of V_H family representation on the VDJ^+ alleles of the λ5T pre-B cells differs from that of normal preB-II cells, it seems not to be identical to that on their VDJ^- alleles (Table 3). It remains to be investigated with a larger number of cells whether these differences are significant. If they are, the changes would also appear to be in part independent of preBcR expression.

V_H Repertoire in Peripheral B Cells of λ5T Mice

λ5T mice produce both B1 and conventional B cells, although at reduced rates (Kitamura et al., 1992; Rolink et al., 1993). It takes a half year to accumulate half the normal number of peripheral, mature B cells. The reactivities of these peripheral B cells to T cell–dependent and –independent antigens are normal. About half of the

Table 4. V_H Representation in VDJ$^+$/VDJ$^-$-Rearranged Splenic B Cells[a] of λ5T Adult Mice

V_H Family	VDJ$^+$ Allele	VDJ$^-$ Allele
7183	3 (12%)	7 (28%)
Q52	1 (4%)	8 (32%)
J558	17 (68%)	5 (20%)
Others	4 (16%)	5 (20%)

[a] Twenty-five single cells were analyzed (from a pool of three spleens).

peripheral mature B cell pool in λ5T mice consists of B1 cells (Kitamura et al., 1992). To exclude differences in V_H representation between B1 and conventional B cells, we excluded the B1 cells from our analysis by using the marker CD23, which could be used as a marker for conventional B cells (Waldschmidt et al., 1991). The structures of the two H chain alleles was determined in single, CD23$^+$ mature B cells by PCR, followed by sequencing of the PCR products. Again, approximately half of the cells were VDJ$^+$/VDJ$^-$ and the other half VDJ$^+$/DJ rearranged (data not shown). The sequence analyses of the VDJ$^+$/VDJ$^-$ mature B cells are summarized in Table 4.

On the VDJ$^-$ alleles, the V_HJ558 family is underrepresented, while the V_H7183 and V_HQ52 families appear overrepresented. Their V_H representation therefore resembles that of the VDJ$^-$ alleles in normal and λ5T pre-B cells. However, in contrast to bone marrow precursor B cells of λ5T mice, which are incapable of expressing preBcRs and which express a V_H repertoire that is biased toward V_H7183 and V_HQ52, splenic mature B cells of these mice now have an expressed V_H repertoire in which the V_HJ558 family is overrepresented and the V_H7183 and V_HQ52 families are underrepresented. The results imply that the skewing of the V_H repertoire of peripheral sIg$^+$ B cells in λ5T mice occurs at the transition from pre-B to B cells and subsequently resembles that of normal splenic B cells. This demonstrates a role for B cell receptors in the process of V_H repertoire changes in λ5T mice.

V_H Repertoire in c-kit$^+$ cμ$^+$ Pre-B Cells of Normal Mice

Although the majority of B220$^+$ c-kit$^+$ preB-I cells are DJ_H rearranged in both H chain alleles, a subpopulation of 10% of these cells have undergone productive V_H-to-DJ_H rearrangements (Rolink et al., 1994; ten Boekel et al., 1995). Hence, these cells could be sorted by FACS into a pool of sIg$^-$ CD45 (B220)$^+$ c-kit$^+$ cμ$^+$ cells (Figure 1B). Data in Figure 1A show the number of c-kit$^+$ cμ$^+$ cells and large preB-II cells present in adult bone marrow of C57BL/6 mice. Both of these populations have their L chain gene loci in germline configuration (ten Boekel et al., 1995). The V_H repertoire of the c-kit$^+$ cμ$^+$ pre-B cells is shown in Table 5A.

Although only a small number of VDJ$^+$/VDJ$^-$ and VDJ$^+$/DJ cells was analyzed, it is apparent that on the (total) VDJ$^+$ alleles, the V_H7183 and V_HQ52 gene families are overrepresented, whereas the V_HJ558 family is underrepresented. In fact, the observed V_H repertoire is very similar to that of B cells early in ontogeny. Hence, the expressed V_H repertoire of c-kit$^+$ cμ$^+$ pre-B cells is different from that on the VDJ$^+$ alleles of large preB-II cells and resembles the V_H repertoire on the VDJ$^+$ alleles of the pre-B cells from λ5T mice. We have also calculated the ratio of productive to nonproductive rearrangements among V_H7183-DJ_H alleles in the c-kit$^+$ cμ$^+$ cells. Of 31 sequenced alleles, 77% were productively V_HDJ_H rearranged, while only 23% were nonproductive (Table 5B). This is in contrast to the findings obtained from the analysis of large preB-II cells, where most (63%) of the V_H7183-DJ_H rearranged alleles were nonproductive. Taken together, these data suggest that the μH chains expressed in the c-kit$^+$ cμ$^+$ pre-B cells have not influenced the establishment of the V_H repertoire seen in large preB-II cells.

Representation of V_H81X in Precursor B Cells of Normal and λ5T Mice

The V_H81X gene has been a hallmark to evaluate changes in V_H repertoire during B cell development. Several reports showed that B cells early in ontogeny express predominantly V_H81X genes, while in the peripheral repertoire of adult mice, V_H81X-containing μH chains are found rarely (Yancopoulos et al., 1988; Malynn et al., 1990; Decker et al., 1991; Huetz et al., 1993).

Table 6 documents the representation of V_H81X on VDJ$^+$ and VDJ$^-$ alleles of precursor B cells of normal and λ5T mice and of peripheral, mature B cells of λ5T

Table 5. V_H Repertoire in c-kit$^+$ cμ$^+$ Pre-B Cells of Normal Mice

(A) V_H Representation in VDJ/(V)DJ-Rearranged c-kit$^+$ cμ$^+$ Pre-B Cells[a] of Normal Adult Mice

	VDJ$^+$/VDJ$^-$ Cells		VDJ$^+$/DJ Cells	Total
V_H Family	VDJ$^+$ Allele	VDJ$^-$ Allele	VDJ$^+$ Allele	VDJ$^+$ Allele
7183	6	3	8	14 (38%)
Q52	2	2	3	5 (14%)
J558	5	7	6	11 (30%)
Others	2	3	5	7 (19%)

[a] Thirty-seven single cells were analyzed (15 VDJ$^+$/VDJ$^-$- and 22 VDJ$^+$/DJ-rearranged cells, from a pool of five femurs).

(B) Analysis of V_H7183-DJ_H–Rearranged Alleles[b] of c-kit$^+$ cμ$^+$ Pre-B Cells of Normal Adult Mice

Configuration of Allele	Number
Productively rearranged VDJs	24 (77%)
Nonproductively rearranged VDJs	7 (23%)

[b] A total of 31 V_H7183-DJ–rearranged alleles were analyzed.

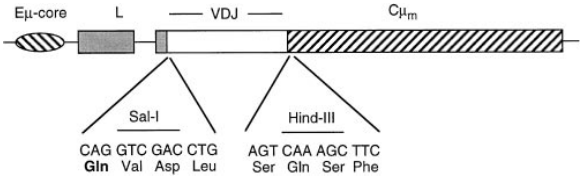

Figure 2. The pELVC Vector

Shown is the part of the vector that contains the leader (L) of the SP6 (Iglesias et al., 1993), a V_HDJ_H-rearranged fragment, and the membrane form of the μH chain constant-region ($C\mu_m$). Expression is controlled by the μ-core enhancer (Eμ-core) upstream of the leader sequence. The first amino acid (Gln) of the V_H domain is shown in bold. A silent HindIII site was created in the third and fourth codons of the Cμm gene. The V_HDJ_H-rearranged fragment can be replaced by another particular V_HDJ_H fragment using the restriction sites SalI and HindIII.

mice. It is evident that in the c-kit$^+$ cμ$^+$ precursors in bone marrow of normal mice and in cμ$^+$ pre-B cells of λ5T mice, V_H81X is present both in the VDJ$^+$ and VDJ$^-$ alleles. In fact, c-kit$^+$ cμ$^+$ pre-B cells showed a V_H81X representation similar to that reported in B cells from fetal liver (Yancopoulos et al., 1988; Marshall et al., 1996). By contrast, in the large preB-II cells of normal mice and in the splenic mature B cells of λ5T mice, V_H81X was not found among the VDJ$^+$ alleles. More strikingly, the frequency of V_H81X-rearranged alleles in the VDJ$^-$ alleles was unchanged in large preB-II cells, that is after the transition of preB-I to preB-II cells. We conclude that expression of a preBcR coincides with the disappearance of V_H81X from the V_H repertoire on the VDJ$^+$ alleles, while in preBcR-defective λ5T mice this disappearance coincides with the expression of the Ig-B cell receptor.

Can μH Chains Expressed in the Repertoire of c-kit$^+$ cμ$^+$ Cells and Large PreB-II Cells Form preBcRs on the Cell Surface?

One mechanism by which the preBcR and the BcR could induce the shift in V_H repertoire in normal and λ5T B lineage cells, respectively, would be that the (surrogate) light chain is not able to associate with some V_H domains of the V_H families V_H7183 and V_HQ52. This could exclude such V_H-expressing pre-B cells from the proliferative expansion or selection into the periphery, since these cells lack surface-bound preBcRs or BcRs, respectively. In fact, two V_H81X-containing μH chains derived from A-MuLV transformed pre-B cell lines have been found not to associate covalently with the surrogate L-chain to form a preBcR that can be deposited in the cell surface membrane (Keyna et al., 1995). Therefore, we designed an assay to determine the capacity of different μH chains to form a preBcR on the cell surface.

Productively V_HDJ_H-rearranged alleles obtained from single-cell PCR analyses were subcloned into the retroviral vector pELVC (Figure 2) and transfected in the SL chain–expressing, cμ$^-$ pre-B cell line 38B9 (Alt et al., 1984), as decribed in Experimental Procedures. The emerging clones were tested for cytoplasmic and cell surface expression of the μH chains. Figure 3 shows a representative analysis of one infected cell line expressing a μH chain in the cytoplasm as well as on the cell

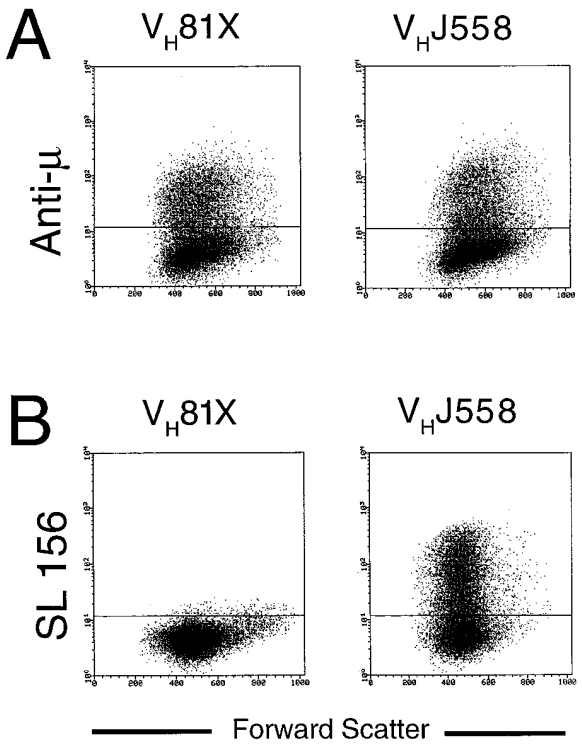

Figure 3. Representative FACS Profile of Two Different μH Chains after Transfection into 38B9 Cells

(A) Expression of V_H81X-containing and V_HJ558-containing μH chains in the cytoplasm.

(B) Detection of cell surface expression of the V_H81X- and V_HJ558-containing μ chain in complex with the SL chain (anti-SL 156) after transfection in the pre-B cell line 38B9. Despite expression in the cytoplasm, the V_H81X-containing μH chain is not expressed on the cell surface, whereas the V_HJ558-containing μH chain is expressed on the cell surface.

surface, and another cell line expressing the transfected μH chain in the cytoplasm but not on the cell surface. Figure 4 provides a summary of these infection analyses with 33 μH chains from c-kit$^+$ cμ$^+$ cells, 21 μH chains from large preB-II cells, and 9 μH chains from λ5T pre-B cells. Surface expression of the transfected μH chains was detected with anti-μ, but similar results were obtained with an antibody (monoclonal antibody [MAb] SL-156) recognizing the μH/SL chain complex (data not shown). Twenty of 21 different μH chains derived from the large preB-II compartment of normal mice are capable of forming a preBcR on the cell surface. However, only 14 of 33 μH chains from the c-kit$^+$ cμ$^+$ pre-B compartment can do so, despite cytoplasmic μH chain expression in all transfectants. We conclude that the large preB-II cells that are selected for μH chain production are capable of forming a preBcR, whereas only half on the μH chains expressed in c-kit$^+$ cμ$^+$ B cells can do so.

Since some of the c-kit$^+$ cμ$^+$ pre-B cells can potentially make a preBcR, it is likely that they are in transit from the preB-I to the large preB-II pool. However, the rest of these cells are unable to express preBcRs on their cell surface. Hence, they are likely not to participate in the proliferative expansion that marks the transition from preB-I to preB-II cells.

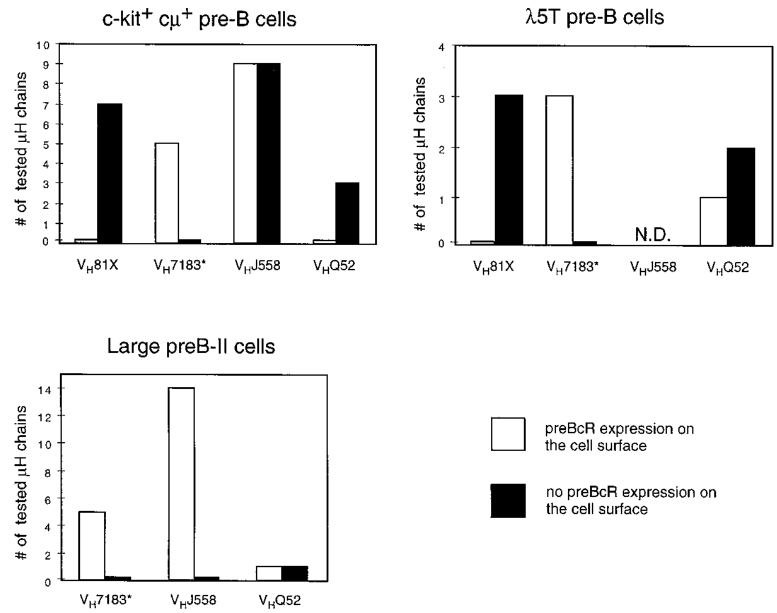

Figure 4. The Capacity of μH Chains Derived from Different Pre-B Cell Compartments to Form preBcRs

Different μH chains derived from c-kit^{+} cμ^{+} pre-B and large preB-II of normal and cμ^{+} sIg^{-} pre-B cells of λ5T mice were transfected into 38B9 cells and examined for their capacity to be expressed on the cell surface. Members of the V_H7183 family, other than V_H81X, are designated as V_H7183*. N.D., not done.

It is striking that all V_H81X-containing μH chains tested from normal as well as from λ5T pre-B cells failed to form a preBcR. In fact, among the V_H7183-containing μH chains tested, the V_H81X gene is the only member of this family that could not form a preBcR on the cell surface (Figure 4). Among the V_HQ52 family, several members appear deficient in forming a preBcR. Figure 5 shows representative amino acid sequences of μH chains that can and cannot form a cell surface preBcR.

Figure 6 provides a summary of the V_H repertoire analyses in combination with the transfection studies. In c-kit^{+} cμ^{+} pre-B cells, about half of the V_H7183-DJ_H rearrangements were found to use V_H81X (Table 6). Since V_H81X appears from our analyses to be the only V_H7183 family member that cannot form a preBcR, this then indicates that about half of the V_H7183-containing μH chains in c-kit^{+} cμ^{+} pre-B cells cannot form a preBcR. Furthermore, about half of the V_HJ558-containing μH

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1                                                                        name
QVDLKESGPGLVAPSQSLSITCTVSGFSLTRYAISWVRQPPGKGLEWLGVIWTGGGTNYNSALKSRLSIS  ck 11.1
QVDLKESGPGLVAPSQSLSITCTVSGFSLTSYGVSWVRQPPGKGLEWLGVIWGDGSTNYHSALISRLSIS  ck 29.5
QVDLKESGPGLVAPSQSLSITCTASGFSLTSYGVDWVRQPPGKGLEWLGVIWGGGSTNYNSALMSRLSIS  L5T VDA
QVDLKESGPGLVAPSQSLSITCTVSGLSLTSYGVHWVRQPPGKGLEWLGVIWAGGSTNYNSALISRLSIS  L5T VDD
QVDLKQSGPGRVAPSQSLSITCTVSGFSLTSYGVHWVRQSPGKGLEWLGVIWRGGSTDYNAAFMSRLSIT  L5T VDE
QVDLKQSGPSLVQPSQSLFITCTVSGFSLTSYGVHWVRQSPGKGLEWLGVIWRGGSTDYNAAFMSRLSIT  TL II7
QVDLKESGPGLVAPSQSLSITCTVSGFSLTSYGVHWVRQSPGKGLEWLGVIWRGGSTDYNAAFMSRLSIT  TL VD12

EDNSKSQVFLKMNSLQTDDTARYYCA|RRGTTVGR--|---FDYWGQGTTLTVSS  ck 11.1    VQ52-DJ2 (D: FL16, RF 2)  SL−
KDNSKSQVFLKLNSLQTDDTATYYCA|KPVGSSYVGD|--YFDYWGQGTTLTVSS  ck 29.5    VQ52-DJ2 (D: FL16, RF 1)  SL−
KDNSKSQVFLKMNSLQTDDTAMYYCA|NPIITTVVAG|----AYWGQGTLVTVSA  L5T VD A   VQ52-DJ3 (D: FL16, RF 2)  SL−
KDNSKSQVFLKMNSLQTDDTAMYYCA|REGDGNYPY-|YYAMDYWGQGTSVTVSS  L5T VD D   VQ52-DJ4 (D: sp2.8, RF 1) SL+
KDNSKSQVFFKMNSLQADDTAIYYCA|KMQRVTR---|---FAYWGQGTLVTVSA  L5T VD E   VQ52-DJ3 (D: sp2.9, RF 2) SL−
KDNSKSQVFFKMNSLQADDTAIYYCA|DDGYYQ----|-TWFAYWGQGTLVTVSA  TL II 7    VQ52-DJ3 (D: sp2.9, RF 1) SL−
KDNSKSQVFFKMNSLQADDTAIYYCA|KTIVPY----|YYAMDYWGQGTSVTVSS  TL VD 12   VQ52-DJ4 (D: sp2.8, RF 1) SL+
71
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1                                                                        name
QVDLQQSGPELVKPGTSVKVSCKASGYAFTNYLIEWVKQRPGQGLEWIGVINPGSGGTNYNEKFKGKATL  CK 1
QVDLQQSGPELVTPGASVKMSCKASGYTFTSYWITWVKQRPGQGLEWIGDIYPGSGSTNYNEKFKSKATL  CK 6
QVDLQQSGPELVKPGASVKLSCKASGYTFTSYWMHWVKQRPGQGLEWIGEIYPNSGRTNYNEKFKGKATL  CK 12
QVDLQQSGPELVKPGTSVKLSCKASGYTFTSYWMHWVKQRPIQGLEWIGNIDPSDSETHYNQKFKDKATL  CK 29
QVDLQQSGPELVKPGASVKMSCKASGYTFTTYPIEWMKQNHGKSLEWIGNFHPYNDDTKYNEKFKGKATL  CK 41

TADKSSSTAYMQLSSLTSEDSAVYFCA|R--------PPGG|-----YWGQGTTLTVSS  CK 1   VJ558-DJ2 (D: ?, RF ?)     SL+
TVDTSSSTAYMQLSSLTSEDSAVYYCA|S------KLLRYP|YWYFDVWGTGTTVTVSS  CK 6   VJ558-DJ1 (D: FL16, RF 3)  SL+
TADTSSSTAYMDLSSLTSEDSAVYYCA|SIWDGSITTGQPL|----DYWGQGTTLTVSS  CK 12  VJ558-DJ2 (D: FL16, RF 1)  SL+
TVDKSSSTAYMQLSSLTSEDSAVYYCA|R----------SR|-------WGQGTLVTVSA  CK 29  VJ558-DJ3 (D: ?, RF ?)     SL−
TVEKSSSTAYLELSRLTSDDSAVYYCA|R---------PRT|--YFDVWGTGTTVTVSS  CK 41  VJ558-DJ1 (D:ST-4, RF 1)   SL−
71
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Figure 5. Amino Acid Sequence of Isolated V_HQ52 and V_HJ558-DJ_H Rearrangements

Amino acid sequence is shown in one-letter code. V_HDJ_H domains of H chains that can and cannot form a cell surface–expressed preBcR are designated as SL^{+} SL^{-}, respectively. The region between the V_H and J_H domains is boxed. The H chains are derived from c-kit^{+} cμ^{+} pre-B cells (ck), large preB-II cells (TL) of normal mice, and cμ^{+} pre-B cells of λ5T (L5T) mice.

Table 6. Frequency of V_H81X among All V_HDJ_H- and V_H7183-DJ_H–Rearranged Alleles[a] in Different B Cell Compartments of Normal and λ5T Adult Mice

	V_H81X			
	Among All V_HDH_H Rearrangements		Among V_H7183 Rearrangements	
B Cells	VDJ^-	VDJ^+	VDJ^-	VDJ^+
c-kit^+ $c\mu^+$	13% (2/15)	19% (7/37)	71% (5/7)	46% (11/24)
Large preB-II	14% (7/51)	0% (0/100)	47% (7/15)	0% (0/9)
λ5T BM	19% (8/42)	15% (14/92)	53% (8/15)	64% (14/22)
λ5T SPL	8% (2/25)	0% (0/25)	30% (2/7)	0% (0/3)

[a] Data obtained from VDJ^+/VDJ^-- and VDJ^+/DJ-rearranged cells (Tables 2–4), with the exception of c-kit^+ $c\mu^+$ cells (Tables 5A and 5B). BM, bone marrow, $c\mu^+$ pre-B cells; SPL, splenic, mature B cells.

chains in this precursor pool cannot form a preBcR. It has not yet been determined whether these V_HJ558 genes are a special subset among the whole V_HJ558 family.

These analyses show that V_H-segments of all three V_H families tested, V_H7183, V_HQ52 and V_HJ558, can participate in productively rearranged alleles that produce μH chains incapable of forming a preBcR. It remains to be determined which of the V_H domains analyzed here show the lack of association with the SL chain as a result of structural restriction of the V_H-encoded portions of the domain, and which of them have a CDR3 domain that restricts pairing.

Discussion

In the present investigation we studied the V_H repertoire of B-lineage cells at different developmental stages in the bone marrow of adult and λ5T mice. Since we performed the analyses at the single-cell level, we were able to distinguish the V_H repertoire on the productively and the nonproductively rearranged H chain alleles in parallel. Furthermore, the expressed V_H repertoire was tested for the capacity to form preBcR expression on the cell surface after transfection in an A-MuLV transformed pre-B cell line. Although many V_H families are included in our results, we provide a comprehensive analysis of the representation of the three most frequently used V_H families during B cell development: V_H7183, V_HQ52, and V_HJ558.

We have demonstrated that the V_H repertoire on the productively rearranged H chain alleles is biased toward the D-proximal V_H7183 and V_HQ52 families, and especially toward the V_H81X segment in c-kit^+ $c\mu^+$ pre-B cells of normal mice and in $c\mu^+$ pre-B cells of λ5T mice. In contrast, in large preB-II cells of normal mice as well as peripheral mature B cells of λ5T mice, the D-proximal V_H families' representation was found suppressed on the productively but not on the nonproductively V_HDJ_H rearranged alleles. This was particularly so for the V_H81X gene and for some of the V_HQ52 genes.

All of the tested V_H81X- and a large proportion of V_HQ52-containing μH chains derived from the expressed V_H repertoire of c-kit^+ $c\mu^+$ pre-B cells of normal mice and $c\mu^+$ pre-B cells of λ5T mice proved incapable of forming a preBcR. About half of the V_HJ558-containing μH chains also were found not to be able to form preBcRs. On the other hand, virtually all μH chains derived from large preB-II cells of normal mice were capable of forming a preBcR. These data show that the shift in the V_H repertoire is mediated by the preBcR.

To interpret our results and to compare them to previous studies made in other laboratories, it is helpful to consider the sizes and lineage relationships of the $sIgM^-$ B-lineage precursors in the bone marrow of normal and λ5T mice (Figure 1A). We have previously been able to separate B-lineage precursors according their cell cycle status and their differential expression of c-kit and CD25. Single-cell PCR analyses of the rearrangement status of H and L chain alleles have allowed us to define a sequence of precursor compartments in normal bone marrow beginning with cycling c-kit^+ $CD25^-$, then cycling c-kit^- $CD25^+$, and finally resting c-kit^- $CD25^+$ cells, named preB-I, large preB-II, and small preB-II, respectively (ten Boekel et al., 1995). The preB-I pool also expresses CD43, a marker used by others to separate B cell precursors (Hardy et al., 1991). PreB-I cells have their L chain genes in germline configuration and are mainly DJ_H rearranged on both H chain alleles. Occasionally, a V_HDJ_H-rearranged allele was found (Rolink et al., 1994; ten Boekel et al., 1995).

The c-kit^+ $c\mu^+$ cells analyzed in this study constitute 10% of the preB-I pool (Figure 1A). A large proportion (~50%) of the μH chains expressed in this subpopulation cannot form a preBcR on the cell surface (Figure 6). If the transition from c-kit^+ pre-B cells to c-kit^- preB-II cells depends on surface expression of a preBcR, then it would be understandable that cells with a productively rearranged locus encoding for a nonpairing μH chain are retained in the preB-I pool. Those cells found in this population expressing a μH chain that can form a preBcR might have undergone this rearrangement so recently that they had not had time to express the preBcR.

In λ5T mice the preB-I pool is 2–3 times the size of this pool in normal mice (Figure 1A). The $c\mu^+$ subpopulation is 3 times the size of the c-kit^+ $c\mu^+$ pool in normal mice. More than 90% of the $c\mu^+$ pre-B cells in λ5T mice are in fact c-kit^+ (Figure 1A) and also express CD43 (unpublished data). Again, a large proportion (~50%) of the μH chains expressed in these compartments cannot pair with the SL chain, although the V_HJ558-μH chains have not been analyzed. Hence, the $c\mu^+$ preB-I population of normal and λ5T mice appear to have a similar composition, although they differ in size.

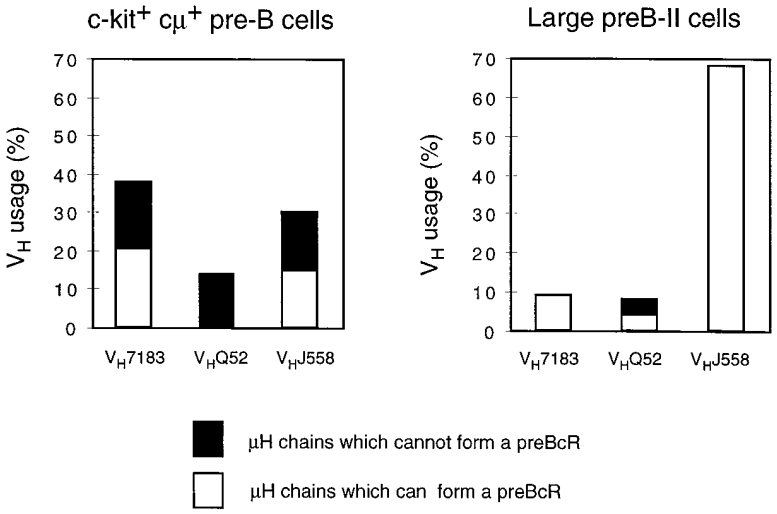

Figure 6. Summary of the V_H Repertoire Analyses

Representation of the V_H families in productively V_HDJ_H-rearranged alleles of c-kit$^+$ cμ$^+$ pre-B cells and large preB-II cells (data derived from Tables 2, 5A, and 6), showing the capacity of the μH chains to form preBcRs (data extrapolated from Figure 4).

None of the pre-B cells in λ5T mice can express a preBcR, regardless of whether they have managed to produce an SL chain–pairing or a nonpairing μH chain. Hence, none of them enters the phase of proliferative expansion that fills the large and small preB-II cell compartments. Since the bone marrow of λ5T mice is essentially devoid of the subsequent pools of large and small preB-II cells, precursors appear to pile up at the transition from the preB-I to more mature stages of cells. The data presented in Figure 1A and the finding that approximately 90% of the pre-B cells have their L chain loci in germline configuration in fact suggest that the block in B cell development in λ5T mice occurs right after V_HDJ_H rearrangements.

We have found previously that these λ5T preB-I cells are normally capable of differentiation to sIgM$^+$ cells but do so without proliferative expansion and hence at a much lower rate than normal preB-I cells (Rolink et al., 1993). This means that whenever L chain genes are rearranged and expressed, they can promote the development of immature sIgM$^+$ B cells if the μH chains can pair with the L chain to form a cell surface–bound BcR.

Our analyses of the V_H repertoire in peripheral sIg$^+$ B cells of λ5T mice indicate that conventional L chains in B cell receptors can perform the function of the (missing) SL chain in the V_H repertoire establishment. The shift from a biased V_H repertoire in pre-B cells to a "suppressed" repertoire in peripheral sIg$^+$ B cells in λ5T mice demonstrates that cells expressing D-proximal V_H gene family–containing μH chains can also be lost at later stages of B cell development. Since we found that the representation of V_H7183 and V_HQ52 families is suppressed in sIgM$^+$ B cells of λ5T mice, it is reasonable to assume that the μH chains that cannot pair with SL chains also cannot pair with conventional L chains (Keyna et al., 1995). However, a stringent test of this assumption is still needed.

To conceive a possible scenario in which such a V_H repertoire change could take place, one should remember that B-lineage cells without sIg expression are absent from the peripheral, mature B cell pool. Whatever the selection pressure may be that allows only sIg$^+$ and not sIg$^-$ cells to enter the periphery, it might be the reason for the lack of cells with μH chains that cannot pair with L chains to enter the peripheral B cell pool in λ5T mice.

In the bone marrow of normal mice, large preB-II cells appear to develop from preB-I cells whenever the DJ_H rearranged cells undergo a productive V_HDJ_H-rearrangement and produce an SL chain–pairing μH chain. Approximately one fifth of the large preB-II cells express a preBcR on their cell surface. These preBcR$^+$ cells have their L chain loci in germline configuration and do not transcribe them. The other 80% of this large preB-II pool no longer express the preBcR, since they have downregulated the expression of the SL-chain genes. This preBcR$^-$ subpopulation appears to develop from the preBcR$^+$ cells and also appears to constitute the precursors of the small preB-II cells, since they begin to transcribe the L chain loci but have not yet begun L chain rearrangements (Grawunder et al., 1995). A portion of the large preB-II cells express CD43 and most likely are the preBcR-expressing cells (Rolink et al., 1994).

In the pool of large preB-II cells of normal mice we found that the V_H representation of V_H7183 and V_HQ52 families is suppressed. Hence, this suppression is established at a very early stage of B cell development.

If we were to look for any overexpression of the D-proximal V_H families in the total pools of all sIg$^-$ precursors in bone marrow, we would need to look for 3×10^5 among a total of 250×10^5 cells (Figure 1A). We would need to see all protein and DNA analyses and distinguish 1% overexpression in 99% suppressed productive H chain alleles. Previously, V_H repertoire analyses of bone marrow B cell precursors have led to conflicting conclusions. A biased V_H repertoire was reported in bone marrow B cells of BALB/c mice, although the repertoire was less biased compared to B cells early in ontogeny (Freitas et al., 1990; Malynn et al., 1990). In these reports subpopulations of sIg$^-$ precursors expressing markers of early cells were analyzed. These results were confirmed recently in B220$^+$ CD43$^+$ sIg$^-$ cells of bone marrow (Marshall et al., 1996), although other reports failed to confirm this observation (Jeong and Teale, 1988; Jeong and Teale, 1989; Miceli and Schulze, 1991). In addition, no preferential usage of the

most D-proximal V_H families has been found in bone marrow B cells from C57BL/6 mice (Freitas et al., 1990). A likely explanation for the detection of either a slightly biased or unbiased V_H repertoire in these studies may be that the B-lineage cells analyzed in these reports belonged predominantly to the large pool of the total preB-II compartment, which makes up 80%–90% of all precursor B cells.

Taken together, our results show that the suppression of the D-proximal V_H representation in the repertoire of B-lineage cells in bone marrow is established at the transition of the preB-I to preB-II cell stage of differentiation and that it involves the surface expression of preBcR. The loss, or "suppression", of V_H81X- and V_HQ52-expressing μH chains appears to be the consequence of their inability to form a preBcR and the inability of the pre-B cells expressing them to participate in proliferative expansion of preB-II cells. Other V_H families, such as the V_HJ558 family, are favored at this stage.

Preferential usage of D-proximal V_H-segments, in particular of V_H81X, has been seen most clearly early in ontogeny, for instance in fetal liver B cell development (Yancopoulos et al., 1984; Perlmutter et al., 1985; Wu and Paige, 1986; Alt et al., 1987). One major difference in B cell development in fetal liver compared with that in bone marrow is the lack of expression of terminal desoxynucleotidyl transferase and hence the lack of N-region insertions into the V_HDJ_H joints in fetal liver B-lineage cells (Rolink and Melchers, 1991). Recently, Marshall et al. (1997) established an "in vitro" differentiation culture system in which they studied changes in the V_H repertoire of early fetal liver (day 10 of gestation) and $B220^+$ $CD43^+$ $sIgM^-$ early bone marrow precursors during growth on stromal cells in the presence of IL-7 and, subsequently, during differentiation to $sIgM^+$, lipopolysaccharide (LPS)–stimulated B cells after removal of IL-7. In the first phase of these cultures, on stromal cells in the presence of IL-7, preB-I cells are expected to proliferate as DJ_H-rearranged cells whereas all productively V_HDJ_H-rearranged cells are expected to stop growing (Rolink et al., 1991). The few V_HDJ_H-rearranged cells of both fetal liver and bone marrow at the end of their first culture period preferentially used V_H81X; they appeared to be caught at a very early stage in V_HDJ_H-rearrangements. Striking differences were seen when differentiation to $sIgM^+$, LPS-stimulated cells was induced. V_H81X usage in the productive alleles was suppressed in bone marrow B cells, but not or at least far less in fetal liver B cells. Given our results, we suggest an explanation for these intriguing findings, which Marshall et al. (1997) could not provide because they did not test the V_H81X-containing μH chains for pairing capacity with SL chains and with L chains. We suggest that most B-lineage cells from bone marrow express V_H81X-μH chains with N-regions that cannot pair with the SL chain and L chains. Consequently, when L chain genes are rearranged and expressed during differentiation in the absence of IL-7 they will not be able to form an $sIgM^+$ B cell capable of being stimulated by LPS (Rolink et al., 1993). On the other hand, V_H81X-μH chains from fetal liver without N-region insertions should be capable of forming a preBcR and consequently also a BcR and hence should yield $sIgM^+$ B cells capable of being stimulated by LPS. In fact, Martin et al. (1997) have shown that an N^- V_H81X-μH chain can pair with the SL chain and L chains and perform normally as other transgenic μH chains expressing other V_H-segments in mouse B cell development. We are now in the process of testing this hypothesis.

The structural reasons for the inability of certain μH chains to associate with the SL chain and L chains remains to be elucidated. It is, however, clear that some V_H segments, such as V_H81X, tolerate little CDR3 diversity insertion, while others, such as V_HJ558 family members, allow diverse CDR3 regions.

Do pre-B cells expressing a surface preBcR that cannot participate in the proliferative expansion of large preB-II cells exist in normal mice? This possibility cannot be ruled out, since the ratio of V_HJ558- to V_H7183-expressing μH chains that can form a preBcR is approximately 1 in c-kit^+ $c\mu^+$ cells, but 7 in preB-II cells (Figure 6). It implies that the suppression of the V_H7183 family as found in large preB-II cells cannot be explained by the differences of the V_H7183 and V_HJ558 family members in forming preBcRs. Instead, it suggests that pre-B cells bearing these particular V_H7183-containing μH chains participate less efficiently in proliferative expansion than do those containing V_HJ558 segments.

The μH chain–expressing pre-B cells in λ5T mice are in a $preBcR^-$ state, whether or not the μH chains can pair with the SL chain. Selection of V_H repertoires due to differences in proliferative expansion should not occur, and hence all possible V_H segments are expected to be equally represented in the $c\mu^+$ pool. However, we find that the ratio of V_H7183 to V_HJ558 is 15 to 11 in VDJ^- alleles and 10 to 20 in VDJ^+ alleles, and thus is shifted toward V_HJ558 in VDJ^+ alleles (Table 3). Although the limited numbers of cells tested so far do not allow strong conclusions, they are consistent with the findings of Marshall et al. (1996), who observed a decline in V_H81X usage during fetal B cell development in mice that are incapable of depositing preBcRs and BcRs on the cell surface, that is, in μMT mice. Marshall et al. (1996) proposed two models to account for this change, models that also are applicable to the V_H repertoire changes that we have observed and that cannot be explained by preBcR or BcR expression. In their view, these changes might be explained first by changes in the probabilities of use of different V_H segments for V_HDJ_H rearrangements during different stages of B cell development. However, since we found that the V_H repertoires on the VDJ^- versus VDJ^+ alleles differ and that the expressed repertoire in VDJ^+/DJ cells is similar to that of VDJ^+/VDJ^- cells, it is unlikely that the V_H-shift is caused by this mechanism. Alternatively, these changes might be the result of replacement of the V_H segment in V_HDJ_H rearrangements, by an upstream V_H segment. Such V_H replacement reactions were first seen in pre-B cell lines (Kleinfield et al., 1986; Reth et al., 1986a; Takemori et al., 1987; Covey et al., 1990; Shirasawa et al., 1992) and have also been observed recently in H chain-transgenic mice, in which a V_HDJ_H rearranged gene was introduced into the H chain gene locus at the homologous site (Chen et al., 1995; Taki et al., 1995; Cascalho et al., 1996).

Is the c-kit^+ $c\mu^+$ subpopulation of the preB-I pool of normal mice and the $c\mu^+$ pool of λ5T mice a "dead end" for all of the cells that produce a μH chain that cannot

pair with the SL chain and L chain, and hence, cannot make a preBcR or BcR? These cells might be in a state allowing rearrangement and V_H gene replacement, by keeping the rearrangement machinery active. It has been suggested that expression of the preBcR on the cell surface delivers a signal that down-regulates RAG-1 and RAG-2 proteins and therefore prevents ongoing rearrangements on the H chain loci (Grawunder et al., 1995). In λ5T mice, this signaling could be done by the BcR. It is plausible that V_H gene replacements can take place as long as the rearrangement machinery is operating, that is, as long as there is no preBcR deposition on the cell surface. We demonstrate in this report that all V_H81X-containing μH chains, half of the V_HJ558-containing, and most of the V_HQ52-containing μH chains found in the c-kit$^+$ cμ$^+$ pre-B cell pool of normal and λ5T mice fail to form a preBcR. Thus in these cells of normal and in all pre-B cells of λ5T mice, the H chain locus might remain susceptible to ongoing rearrangements, such as V_H-gene replacements. Since V_H81X is used in remarkably high proportion, it is possible that V_H81X is a preferred substrate for V_H gene replacements, as has been previously suggested (Marshall et al., 1996). However, if V_H gene replacement is a major mechanism causing the V_H repertoire shift, then our results indicate that such V_H replacements occur only on the productively V_HDJ_H rearranged alleles, since suppression of V_H81X usage was not found in the nonproductively V_HDJ_H rearranged alleles. In conclusion, pre-B cells expressing an SL-chain–nonpairing μH chain could be rescued by V_H gene replacements that lead to an SL (or L)–chain pairing μH chain. This would allow these cells to enter proliferative expansion and thus to participate in the preB-II cell pool. It remains to be determined how many B cells go through a state in which μH chains cannot pair with the SL chain—in other words, whether this nonpairing state is mandatory for further B cell development or is just a sideline.

In conclusion, analyses of the V_H repertoire in different pre-B cell compartments of normal mice compared with the V_H repertoire in pre-B cells and splenic sIg$^+$ B cells of λ5T mice reveals that the preBcR is shaping the V_H repertoire at the transition from preB-I to preB-II cells and suggests that the SL chain positively selects μH chains encoded by V_H families that predominate in the later stages of B cell development and that the BcR does the same at the transition from pre-B to immature B cells in λ5T mice.

Experimental Procedures

Mice

C57BL/6 mice were obtained from the Institut fuer Biologisch-Medizinische Forschung AG (Fuellinsdorf, Switzerland). Homozygous λ5T (Kitamura et al., 1992) mice were bred at the Institute's animal facilities. λ5T mice were backcrossed onto C57BL/6 for three generations and selected for homozygous Ig^b allotype. Mice were analyzed at 6–12 weeks of age.

FACS Staining and Sorting of Single Cells

Cell Surface Staining

Cells for FACS sorting were prepared from bone marrow and spleen as described before (Rolink et al., 1994). The MAbs used were obtained from PharMingen (San Diego, CA). Bone marrow cells were depleted of sIgM$^+$ B cells using sheep anti-mouse Ig-conjugated Dynabeads (Dynal AS, Skćyen, Norway) and stained with fluorescein isothiocyanate (FITC)–conjugated MAb RA3 6B2 (anti-CD45R, B220) and double-stained with biotin-conjugated MAb ACK-4 (anti-c-kit) (Ogawa et al., 1991) or 7D4 (anti-CD25, TAC). To obtain cμ$^+$ cells from the B220$^+$ c-kit$^+$ compartment, B220$^+$ c-kit$^+$ cells were sorted after depletion of sIg$^+$ B cells and subsequently restained for cytoplasmic μH chain expression (see below and Figure 1B). Splenic mature B cells were sorted as B220$^+$ CD23$^+$ cells using FITC-conjugated MAb RA3 6B2 and biotin-conjugated MAb B3B4 (anti-CD23, IgE Fc-receptor). Binding of biotin-conjugated MAb was visualized using streptavidin-phycoerythrin (Southern Biotechnology Associates, Birmingham, AL).

Intracellular Staining

Cytoplasmic μH protein expression was detected by fixation of 10^6 cells in 2% paraformaldehyde (Fluka Chemie Ag., Buchs, Switzerland) dissolved in phosphate-buffered saline (PBS) for 20 min at room temperature. Subsequently the cells were washed two times in PBS and incubated with FITC-conjugated polyclonal anti-IgM (Southern Biotechnology Associates) in 0.5% saponin (Sigma Chemie, Buchs, Switzerland) for 30 min at room temperature. Then cells were washed once in 0.5% saponin and once in PBS supplemented with 2% fetal calf serum (FCS) and analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

Sorting of Single Cells

Cells were sorted using the FACStar Plus equipped with an automatic cell deposition unit (Becton Dickinson). Single cells were directly sorted into 96-well plates (Costar, Cambridge, MA) containing 7 μl of H_2O and 3 μl of PCR buffer (0.5 mM 2-β mercaptoethanol, 0.5 M KCl, 0.1 M Tris-HCl [pH 8.3], 15 mM $MgCl_2$, and 0.01% gelatin). To prepare DNA, 2 μl of proteinase K (5 mg/ml; Boehringer Mannheim, Mannheim, Germany) was added and samples were overlaid with PCR oil (Fluka). The samples were digested for 1 hr at 55°C followed by 10 min of incubation at 95°C to inactivate the proteinase K. Plates were then stored at −70°C until use for PCR analysis.

PCR Analysis of Ig H Chain Gene Rearrangements

DNA amplification was carried out in two rounds of PCR using a Hybaid Omnigene PCR machine (Hybaid, Middlesex, UK). PCR conditions and sequences of the primers were as described before (ten Boekel et al., 1995). The first round of amplification contained two different 5′ D primers ($D_{FL/SP}$ and D_{Q52}) and six different 5′ V_H primers recognizing together the families V_HJ558, V_H7183, V_HQ52, V_HJ606, V_HS107, V_HX24, V_H36–60, V_HDNA-4, and V_HGAM3.8, in combination with a 3′ primer downstream of J_H4. In the second round of PCR, 1 μl of the first PCR amplification was reamplified with a V_H family–specific or a D primer and a nested J_H4 primer.

DNA Sequencing of V_HDJ_H and DJ_H Rearrangements

The products of the second PCR round were sequenced using the Ready Reaction Dye Determinator (Perkin Elmer, Forster City, CA) and the automated DNA sequencer 373 (Applied Biosystems, Forster City, CA). The following primers were used: (J_H4) 5′ GGG TCT AGA CTC TCA GCC GGC TCC CTC AGG G, (J_H3) 5′ CCA GAC CCA TGT CTC AAC TTT GGG AC, (J_H2) 5′ AGG TGT CCC TAG TCC TTC ATG ACC TG, and (J_H1) 5′ GCA GAG TGT GGC AGA TGG CC for sequencing of (V_H)DJ_H rearrangements involving J_H4, J_H3, J_H2, and J_H1, respectively.

Construction of pELVC and Subcloning of V_HDJ_H PCR Fragments

The pELVC was derived from the pLXSP vector described previously (Bäckström et al., 1996) and contained, respectively, the leader sequence of SP6 (Iglesias et al., 1993) and the membrane form of the μH chain constant region. Expression of the μH chain is controlled by the μH chain core enhancer.

V_H7183-DJ_H–rearranged DNA samples obtained after the first round of single-cell PCR were amplified in a second PCR round using the V_H primer (5′ TGC GAG GTC GAC CTG GTG GAG TCT GGG 3′) in combination with the J_H-Cμ primer (5′ TTT GGG AAG CTT TGA CTC TCT GAG GAG ACT/G GTG 3′). In the case of V_HJ558 and V_HQ52-DJ_H rearrangements, the second round included a mixture of two V_H primers: 5′ TGC GAG GTC GAC CTG CAA CAG TCT GGA CCT 3′ and 5′ TGC AAC AGT CTG GAC CTG AGC TGG TGA

AGC CTG GGG CTT CAG 3′ for V_HJ558, and 5′ TGC CAG GTC GAC CTG AAG CAG TCA GGA CCT 3′ and 5′ TGA AGC AGT CAG GAC CTG GCC TGG TGG CGC CCT CAC AGA GCC TGT CC 3′ for V_HQ52. The PCR fragments were subcloned in the pELVC vector using the SalI and HindIII sites (Figure 2).

Transfection and Infection of Pre-B Cells

GP^+E-86 cells (2–2.5 × 10^6) (Chen and Okayama, 1987) seeded 5 hr previously in dishes (60 mm) in 5 ml of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS were transfected with 5–10 μg of retroviral plasmid DNA using the calcium phosphate method decribed earlier (Pear et al., 1993). The medium was removed after overnight incubation (37°C, 5% CO_2) and 5 ml of fresh IMDM-10% FCS culture medium was added. Following a further 24 hr incubation, the virus-containing supernatant was harvested from the culture dishes and filtered through a 0.45 μm filter. An aliquot of 2 ml of this supernatant supplemented with 4 μg/ml polybrene (Sigma Chemie) was used to infect 5 × 10^5 38B9 cells (A-MuLV transformed pre-B cell line; Alt et al. 1984) in a 5 ml dish. After incubation for 5 hr, 5 ml of IMDM supplemented with 2% FCS was added. The next day, infected B cells were selected by adding 3 μg/ml puromycin (Sigma Chemie).

Flow Cytometry Analysis of μH Chain–Transfected Pre-B Cells

Intracellular Staining

Testing of cytoplasmic μH chain protein expression was performed as decribed above.

Cell Surface Staining

Detection of surface μH chain expression was performed by incubating 10^5 cells with biotin-conjugated mAB SL156 (anti-μH/SL chain complex; Winkler et al., 1995) or FITC-conjugated polyclonal anti-IgM in PBS supplemented with 2% FCS. Dead cells were excluded using 0.2 μg/ml propidium iodide (Sigma Chemie).

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